

EXHIBIT 2

Native Oligomeric Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Elicits Diverse Monoclonal Antibody Reactivities

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We synthesized and purified a recombinant human immunodeficiency virus type 1 (HIV-1) envelope (Env) glycoprotein, lacking the gp120/gp41 cleavage site as well as the transmembrane domain, that is secreted principally as a stable oligomer. Mice were immunized with separated monomeric and oligomeric HIV-1 Env glycoproteins to analyze the repertoire of antibody responses to the tertiary and quaternary structure of the protein. Hybridomas were generated and assayed for reactivity by immunoprecipitation of nondenatured Env protein. A total of 138 monoclonal antibodies (MAbs) were generated and cloned, 123 of which were derived from seven animals immunized with oligomeric Env. Within this group, a significant response was obtained against the gp41 ectodomain; 49 MAbs recognized epitopes in gp41, 82% of which were conformational. The influence of conformation on gp120 antigenicity was less pronounced, with 40% of the anti-gp120 MAbs binding to conformational epitopes, many of which blocked CD4 binding. Surprisingly, less than 7% of the MAbs derived from mice immunized with oligomeric Env recognized the V3 loop. In addition, MAbs to linear epitopes in the C-terminal domain of gp120 were not obtained, suggesting that this region of the protein may be partially masked in the oligomeric molecule. A total of 15 MAbs were obtained from two mice immunized with monomeric Env. Nearly half of these recognized the V3 loop, suggesting that this region may be a less predominant epitope in the context of oligomeric Env than in monomeric protein. Thus, immunization with oligomeric Env generates a large proportion of antibodies to conformational epitopes in both gp120 and gp41, many of which may be absent from monomeric Env.

The human immunodeficiency virus type 1 (HIV-1) envelope (Env) glycoprotein is a structurally and functionally complex integral membrane protein that plays a number of important roles in virus infection. In addition, it is a target of humoral and cell-mediated immune responses (48, 57, 71). Env is synthesized as a polyprotein precursor, gp160, which is cleaved during transport into gp120 and gp41 subunits (1, 80, 83). The external gp120 subunit is noncovalently associated with gp41 and is responsible for binding virus to CD4 molecules which serve as the cellular receptor (12, 42). The gp41 subunit is anchored in the membrane by virtue of a hydrophobic transmembrane domain (23, 29). Following specific receptor binding, the Env glycoprotein presumably undergoes a conformational change and initiates fusion between the viral envelope and the target cell through the action of the fusion domain of the gp41 molecule (4, 44).

Prior to cleavage, gp160 molecules assemble into noncovalently associated dimers and a higher-order structure, most likely consisting of a dimer of dimers (14, 66, 73). The ectodomain of gp41 is largely responsible for subunit assembly (14, 19, 78). Oligomerization of the Env glycoprotein has several consequences. First, it is required for transport from

the endoplasmic reticulum; mutants that fail to oligomerize are retained and degraded (84). Second, multimeric CD4 binding by Env oligomers may facilitate viral entry (15, 49, 54). Third, as for some other viral proteins (82), Env quaternary structure may have important antigenic implications (6, 66, 68, 76).

There are several reasons to suspect that the oligomeric nature of Env may influence its antigenic structure. Multimeric proteins typically interact over large areas, making structural differences between oligomeric and monomeric subunits likely (39). In the case of HIV-1 Env, nearly the entire gp41 ectodomain is required for stable subunit association (19). Consequently, it is not surprising that some antibodies react more strongly with oligomeric than monomeric gp41 (6, 66, 68). There is also evidence for contacts between adjoining gp120 subunits (19, 62, 81). Antibodies to conformation-dependent epitopes in gp120 are prevalent in the sera of infected individuals and account for the majority of the neutralizing activity (35, 53, 76). In some instances, a significant amount of this neutralizing activity cannot be accounted for by antibodies directed against epitopes in monomeric gp120 (76). Thus, epitopes contingent upon Env quaternary structure to which neutralizing antibodies are directed may exist not only in gp41 but in gp120 as well.

In this report, we describe the production and purification of secreted oligomeric HIV-1 Env protein. Mice were immunized with monomeric, dimeric, or tetrameric HIV-1 Env glycoprotein isolated under nondenaturing conditions to study the effects of Env quaternary structure on the repertoire of antibodies elicited by each form of the protein. Furthermore,

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screening of monoclonal antibodies (MAbs) was performed by immunoprecipitation with the same undenatured Env proteins. The results indicate that HIV-1 Env quaternary structure has profound immunological implications. A large number of MAbs were generated, with more than half recognizing conformational epitopes. We obtained a number of MAbs which block gp120/CD4 binding but relatively few against linear epitopes in the V3 loop. In addition, antibodies against linear epitopes in the C-terminal portion of gp120 were not obtained, suggesting that this region of the protein may be partially masked in the oligomeric molecule. Finally, more than one-third of the MAbs recognized epitopes in the gp41 ectodomain, with 82% of these directed against conformational epitopes. Therefore, immunization with oligomeric Env generates a large fraction of antibodies to conformational epitopes in both gp120 and gp41 and may minimize production of antibodies to variable, linear regions of the protein which react more strongly with monomeric protein.

MATERIALS AND METHODS

Recombinant vaccinia viruses. For production of soluble, secreted HIV-1 Env glycoprotein, two recombinant vaccinia viruses were constructed. The *env* gene from the BH8 isolate (GenBank accession number KO2011) was used (55, 70), and nucleotide numbers are derived from this sequence. Numbering of amino acids begins at the start of the open reading frame and thus includes the signal peptide. For construction of both recombinant viruses, two translation termination codons were inserted after the lysine residue at amino acid 678 (nucleotide 2034), just prior to the transmembrane domain of gp41, using a two-step PCR protocol (38). In the first step, two fragments with overlapping ends were synthesized. These spanned a region of the *env* gene from the *Hind*III restriction site (nucleotide 2128), through the transmembrane coding region, to the *Bam*HI restriction site (nucleotide 2462). One fragment was generated with the synthetic oligonucleotide a (5'-AA CAATTACACAAGCTTAATACACTC-3'), containing the *Hind*III restriction site, in conjunction with oligonucleotide b (5'-CCCCGCGGTTATTATTTATATACCACAGCCA ATTGT-3'), containing the translation termination codons. The other fragment was generated with oligonucleotide c (5'-GTGCTAAGGATCCGTTCACTAATCG-3'), containing the *Bam*HI restriction site, in conjunction with oligonucleotide d (5'-TAATAACCGCGGGGTTATTCATAATGATAGTA GGAGGC-3'). These two fragments were then used together in a second reaction along with oligonucleotides a and c to generate a 372-bp fragment. This fragment was digested with *Hind*III and *Bam*HI and exchanged with the analogous fragment in the *env* gene of pSC60 (7), a plasmid which contains the entire HIV-1 *env* gene under control of a synthetic early/late vaccinia virus promoter. The resulting plasmid, pCB-14, thus contains the *env* gene truncated after amino acid 678. The proteolytic cleavage sites between gp120 and gp41 were removed by substitution of a 575-bp *Ssp*I-*Hind*III fragment between nucleotides 1553 and 2128 with the analogous fragment from pPE12 (17) to generate pPE12B. Plasmid pPE12 contains the *env* gene from which 12 amino acids including the primary and secondary cleavage sites have been removed. Plasmids pCB-14 and pPE12B were used to generate recombinant vaccinia viruses vCB-14 and vPE12B (18), which express cleavable and noncleavable secreted gp140 molecules, respectively.

Several other recombinant vaccinia viruses were also used. vPE16 (16) and vSC60 (7) express wild-type gp160 under the vaccinia virus 7.5K and synthetic early/late promoters, respec-

tively; vPE12 expresses a noncleavable form of gp160; vPE8 expresses gp120; and the series vPE17, vPE18, vPE20, vPE21, and vPE22 (17) express C-terminally truncated Env molecules. vSC64 expresses a chimeric Env glycoprotein molecule consisting of HIV-2 gp120 and HIV-1 gp41 (7). vCB-5 (5) expresses soluble CD4 (sCD4) (372 amino acid residues). In addition, plasmid pPE63 (19), expressing truncated Env glycoprotein via the hybrid vaccinia virus-T7 system (21), was used.

Purification of soluble, secreted HIV-1 Env glycoprotein gp140 for immunizations. Typically, 40 confluent 160-cm² flasks, each containing approximately 1.5×10^7 BS-C-1 cells, were infected with vPE12B at a multiplicity of infection of 10. At 2 h after infection, the monolayers were washed three times with phosphate-buffered saline (PBS) to remove free virus particles and then overlaid with serum-free OPTI-MEM (GIBCO, Grand Island, N.Y.). After 24 to 36 h, the medium was harvested and culture debris was removed by centrifugation for 30 min at 12,000 rpm. Triton X-100 was then added to 0.5% (vol/vol) final concentration and maintained through lentil lectin chromatography in order to prevent nonspecific binding of proteins with the column material. Glycoproteins were purified by lentil lectin-Sepharose (Pharmacia, Piscataway, N.J.) chromatography as follows. The pooled culture supernatant containing secreted gp140 was cycled continuously over a column (1 by 13 cm) overnight. The column was washed with PBS containing 10 mM Tris-HCl (pH 8.0), 0.3 M NaCl, and 0.5% Triton X-100 (10 column volumes) followed by PBS containing 10 mM Tris-HCl (pH 8.0) (2 column volumes). Glycoproteins were eluted with 0.5 M methyl α -D-mannopyranoside in PBS containing 10 mM Tris-HCl (pH 8.0) (3 column volumes) and concentrated 20- to 30-fold in Centricon microconcentrators (Amicon). This material was loaded onto 5 to 20% sucrose gradients in the absence of detergent and centrifuged in an SW40 rotor for 20 h at 4°C at 40,000 rpm (14). After fractionation, a small aliquot of each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting (immunoblotting) using a rabbit polyclonal antiserum to HIV-1 gp160 (R160) (84) and ¹²⁵I-labeled protein A (Amersham). Fractions containing monomeric, dimeric, and tetrameric Env glycoprotein were pooled and concentrated. To verify the oligomeric status of each pooled fraction, aliquots were cross-linked with 1 mM ethyleneglycol bis(succinimidylsuccinate) (EGS; Pierce, Rockford, Ill.) and analyzed by SDS-PAGE (4% gel) and Western blotting with R160 as described previously (14). Purified Env glycoprotein preparations were separated by SDS-PAGE (10% gel) and visualized with Coomassie blue G250.

Immunization of mice and production of hybridomas. A.SW/SnJ mice (Jackson Laboratory, Bar Harbor, Maine) were immunized with either monomeric, dimeric, or tetrameric Env glycoprotein preparations with RiBi adjuvant (RiBi Immunochem Research Inc., Hamilton, Mont.) as recommended by the supplier. Briefly, mice were inoculated at 3-week intervals with 15 to 20 μ g of purified HIV-1 Env glycoprotein per mouse (one-half subcutaneously and one-half intraperitoneally). A test bleed was performed following the first boost, and the sera were assayed by both immunoprecipitating and immunoblotting to ensure reactivity with the immunogen. Mice receiving monomeric and dimeric Env preparations were inoculated three times, while mice receiving tetrameric Env were inoculated four times.

Three days after the final inoculation, mice were sacrificed and the spleens were harvested and prepared for cell fusion by standard methods (22). Splenocytes were fused with Sp2/0 myeloma cells (ATCC 1581) with polyethylene glycol, using a

modification of the method of Gefter et al. (24). Following polyethylene glycol fusion, the cell preparations were distributed in 96-well plates at a density of 10^5 cells per well, based on the number of Sp2/0 partner cells, and selected in Iscove's minimal essential medium supplemented with hypoxanthine-aminopterin-thymidine, 10% fetal calf serum (HyClone Laboratories, Hazelton, Mont.), and 100 U of recombinant interleukin-6 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml. No feeder cell cultures were used. The medium was replaced with fresh hypoxanthine-aminopterin-thymidine medium approximately 10 days after plating. Twelve days after plating, the supernatants from all wells containing a hybridoma colony were screened by immunoprecipitation with iodinated Env glycoprotein preparations. Spleens from the two mice immunized with monomeric Env yielded 378 hybridoma colonies, of which 15 were reactive with Env by immunoprecipitation. Spleens from the three mice immunized with dimeric Env and from the four mice immunized with tetrameric Env yielded 733 and 1,044 hybridoma colonies and 68 and 55 anti-Env MAbs, respectively.

Radioiodination of HIV-1 Env glycoproteins. Preparations (50 μ g) of purified monomeric, dimeric, or tetrameric HIV-1 Env glycoproteins were labeled with 125 I by the chloramine T method with Iodobeads (Pierce Chemical) as described by Markwell (51). Radiolabeled glycoproteins were separated from free iodine by passage over a Sepharose G-25 column and stored at 4°C. Typically, the specific radioactivity of Env glycoprotein was approximately 1×10^6 to 2×10^6 cpm/ μ g. Iodination of Env glycoprotein preparations did not disrupt the oligomeric structure of the soluble Env glycoprotein preparations, as determined by cross-linking with 1 mM EGS and analysis by SDS-PAGE (4% gel) and Western blotting.

Screening of hybridoma supernatants. Hybridoma supernatants were screened by immunoprecipitation. Briefly, 100 μ l of culture supernatant was incubated with 100 μ l of PBS containing 0.5% Triton X-100, 0.5% Nonidet P-40, iodinated Env glycoprotein (approximately 100,000 cpm), and 8 μ g of rabbit anti-mouse immunoglobulin G (IgG; Calbiochem, La Jolla, Calif.) for 1 h at room temperature in microcentrifuge tubes. The oligomeric forms of Env used for screening each set were the same as that used for immunization. Protein A-Sepharose beads (100 μ l of a 20% [vol/vol] suspension) were then added, and tubes were rocked for 30 min. The beads were centrifuged, and the pellets were washed once with PBS containing 0.5% Triton X-100 and 0.5% Nonidet P-40. The tubes were counted in a Beckman Gamma 5500B counter. MAb 902 (10) and polyclonal rabbit antibody R160 (84) were included as positive controls; culture supernatant from an irrelevant hybridoma was included as a negative control.

Hybridoma colonies producing immunoglobulin which immunoprecipitated Env were expanded to approximately 4×10^6 to 5×10^6 cells and cloned by limiting dilution by standard methods. MAbs were produced as tissue culture supernatants in Iscove's minimal essential medium containing hypoxanthine, thymidine, 10% fetal calf serum, and 50 U of interleukin-6 per ml. In some instances, MAbs were produced in serum-free HyQ medium (HyClone) with 50 Units of interleukin-6 per ml. Mouse IgG subclass types were determined by using the AB-STAT typing kit (Sangstat Medical Co., Menlo Park, Calif.). Mouse IgG concentrations in tissue culture supernatants were determined with an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim Biochemicals).

Metabolic labeling and immunoprecipitation. BS-C-1 cells were infected with recombinant vaccinia virus at a multiplicity of infection of 20. At 4 h postinfection, the virus inoculum was replaced with methionine-free minimal essential medium con-

taining 5% dialyzed fetal calf serum and 100 μ Ci of [35 S]methionine per ml and incubated overnight. Cells were lysed in buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.5% Triton X-100. Soluble, secreted forms of Env glycoprotein were obtained from the medium of infected cells. For preparation of the ectodomain fragment of gp41 (gp41s), consisting of the N-terminal 172 amino acids of gp41, the medium of cells infected with vCB-14 was concentrated and applied to a 5 to 20% sucrose gradient as described above. The gp120 and gp41s dissociated from one another during centrifugation, and gp41s was recovered in a single peak sedimenting more slowly than monomeric gp120 (6). Monomeric gp120 was obtained from the medium of infected cells and in some cases was purified by sucrose density gradient centrifugation. Immunoprecipitations were performed by incubating metabolically labeled Env with antibody overnight at 4°C. Typically, 200 μ l of a hybridoma culture supernatant or 1 μ l of a polyclonal antiserum was used for each immunoprecipitation. Where appropriate, 8 μ g of rabbit anti-mouse IgG (Calbiochem) was then added for 30 min followed by 100 μ l of a 20% protein A-Sepharose suspension. After 30 min of rocking, the Sepharose beads were centrifuged at $1,000 \times g$ for 4 min and the pellets were washed twice with 1 ml of Triton buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 0.1% Triton X-100). Proteins were eluted by heating for 5 min at 95°C in sample buffer containing 5% 2-mercaptoethanol.

Western blotting. Proteins from extracts of vaccinia virus-infected cells expressing different HIV-1 *env* genes were separated by SDS-PAGE (10% gel) and transferred to nitrocellulose membranes. In some cases, proteins were separated on preparative gels and the nitrocellulose was cut into 10-mm strips after protein transfer. The nitrocellulose membranes were incubated with MAb (usually 1:5 dilution) for 1 h at room temperature. After washing with PBS containing 0.2% Tween 20, the strips were incubated with 125 I-labeled rabbit anti-mouse IgG for 30 min and then washed. Hybridization with polyclonal antibody R160 was done at 1:500 dilution, and 125 I-protein A was used to detect binding. Proteins were visualized by autoradiography.

Flow cytometry. Human H9 cells ($10^6/50 \mu$ l) chronically infected with HIV-1 IIIB were incubated with hybridoma supernatant. After 30 min at 4°C, the cells were washed twice with PBS containing 1% bovine serum albumin, incubated with goat anti-mouse-fluorescein isothiocyanate for 30 min at 4°C, and then washed two times. The cells were resuspended in 1 ml of PBS containing 4% paraformaldehyde and analyzed with a fluorescence-activated cell sorting (FACS) apparatus (FACScan; Becton Dickinson).

Peptide ELISA. The HIV-1 IIIB V3 loop peptide (CNTR KSIRIQRGPGRAFVTIGK) (American Bio-Technologies, Cambridge, Mass.) and the HIV-1 MN V3 loop peptide (YNKRKRIHIGPGRAFYTITKNIIG) (Biological Resources Branch, National Institute of Allergy and Infectious Diseases [NIAID]) were used to determine the V3 loop reactivities of the MAbs. Briefly, the wells of Immulon II 96-well assay plates were coated with 50 μ l of 0.05 M sodium carbonate (pH 9.5) containing 0.25 μ g of peptide overnight at 4°C. Plates were washed with PBS containing 0.1% Tween 20 and blocked with a solution of proteolyzed gelatin (Boehringer Mannheim Biochemicals). Antibody binding was performed at room temperature for 1 h. Serial dilutions were tested in duplicate. Bound MAb was detected with a peroxidase-conjugated anti-mouse IgG (Boehringer Mannheim Biochemicals) and 2,2'-amino-di-[3-ethylbenzthiazoline sulfonate(6)] substrate (Boehringer Mannheim Biochemicals). All MAbs exhibiting binding were

reexamined on mock-coated plates, and no false positives were detected.

CD4 blocking. To assay for CD4 blocking ability, metabolically labeled gp140 and sCD4 were prepared from the medium of cells infected with vPE12B and vCB-5, respectively. Dimeric gp140 was purified by sucrose density gradient centrifugation as described above. A 100- μ l sample of hybridoma supernatant (MAb in excess of Env) was incubated overnight at 4°C with dimeric gp140. A small amount of sCD4 was added, and the mixture was incubated for 30 min at room temperature. Then 4 μ g of rabbit anti-mouse IgG was added for 30 min, followed by 100 μ l of protein A-Sepharose beads (20% suspension). After 30 min of gentle rocking, the beads were washed once with buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.5% Triton X-100, and samples were analyzed by SDS-PAGE (10% gel). MAb F105 (67), obtained from the AIDS Research and Reference Program, NIAID, was used as a positive control for CD4 blocking activity. Anti-V3 loop MAb 902 (10) was used as a negative control.

RESULTS

Purification of secreted oligomeric Env protein. Secreted oligomeric HIV-1 Env protein was produced by recombinant vaccinia viruses, vCB-14 and vPE12B, containing mutated *env* genes. In both constructs, the genes were truncated after amino acid 678 (Lys), just prior to the transmembrane domain, resulting in 140-kDa Env molecules (gp140). To prevent dissociation of gp120 from the gp41 ectodomain fragment during purification, a deletion was made in the DNA sequence coding for the amino acids at the gp120-gp41 cleavage site in vPE12B. We have previously shown that this mutation, introduced into the full-length *env* gene, yielded a noncleaved form of Env that was efficiently folded, assembled, and transported to the plasma membrane where it could bind to added sCD4 (17). In both constructs, a synthetic early/late vaccinia virus promoter was used to allow high levels of gene expression (7). Both proteins were efficiently secreted and could bind CD4 (not shown), suggesting that they were folded correctly. As designed, the protein made by vCB-14-infected cells was secreted in both cleaved and noncleaved forms, while that expressed by vPE12B was recovered primarily as noncleaved gp140. Although the primary and secondary proteolytic cleavage sites of Env were removed in vPE12B, a very small amount of cleavage occurred, as was observed with the full-length form (17).

To produce oligomeric gp140 for immunization, BS-C-1 monolayers were infected with vPE12B and incubated 24 to 36 h in serum-free medium. The gp140 was purified from the medium by a two-step procedure. First, glycoproteins from the medium were bound to a lentil lectin column and eluted with methyl α -D-mannopyranoside. This step resulted in elimination of most contaminating proteins (Fig. 1C, lane 2). After concentration, oligomeric and monomeric gp140 were separated by sucrose velocity gradient sedimentation. The gradients were fractionated, and a small aliquot of each fraction was analyzed by SDS-PAGE and Western blotting to monitor the distribution of gp140 (Fig. 1A). The majority of the Env glycoprotein was in dimeric and tetrameric forms. A minor peak containing monomeric gp140 and gp120 was also obtained.

Peak fractions containing the three forms were pooled separately, and an aliquot of each was chemically cross-linked with EGS to confirm their oligomeric states. As shown in Fig. 1B, dimeric and tetrameric Env fractions were cross-linked into dimers and larger forms, respectively, whereas monomeric

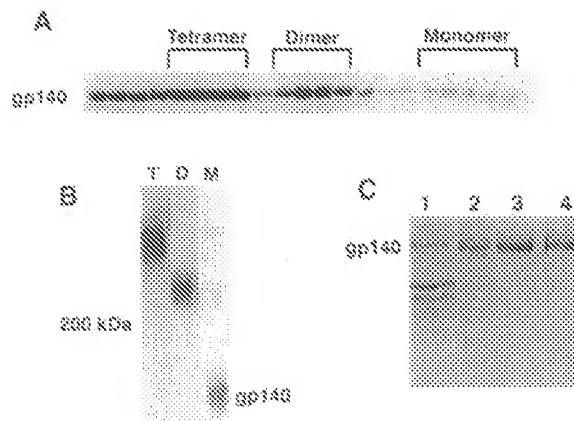


FIG. 1. Purification of monomeric and oligomeric forms of soluble, noncleaved gp140. (A) After separation by lentil lectin chromatography, glycoproteins from the medium of vPE12B-infected cells were separated by sucrose velocity gradient centrifugation. A portion of each gradient fraction was separated by SDS-PAGE (10% gel) and analyzed by Western blotting. Tetramer, dimer, and monomer fractions, as indicated, were pooled. (B) An aliquot of each pool was cross-linked with EGS, separated by SDS-PAGE (4% gel), and analyzed by Western blotting. (C) Proteins from each purification step were analyzed by Coomassie blue staining. Lanes: 1, medium of infected cells; 2, lentil lectin eluate; 3 and 4, dimer and tetramer fractions, respectively, from sucrose density gradient.

Env was not cross-linked into larger-molecular-weight species. Similar results have been demonstrated with full-length Env (14). Analysis of the Coomassie blue staining pattern of each sucrose gradient peak indicated that gp140 was the predominant band in all three preparations, although monomeric Env was less pure than either oligomeric form (Fig. 1C and data not shown).

Immunization of mice and production of MAbs. Two mice were immunized with monomeric, three were immunized with dimeric, and four were immunized with tetrameric gp140. We found that emulsification of the preparations in RiBi adjuvant did not affect the oligomeric state of the Env, as shown by chemical cross-linking followed by Western blot analysis (not shown). Serum collected from each animal after the first inoculation efficiently reacted with gp140 by immunoprecipitation and with gp160, gp120, and gp41 by Western blotting (not shown). The mice were sacrificed 3 days after the final inoculation, and hybridomas were generated. To identify antibodies capable of recognizing conformation-dependent epitopes, hybridoma supernatants were tested for the ability to immunoprecipitate iodinated, gradient-purified oligomeric or monomeric gp140. The form of Env used for screening was the same as the form used for immunization. The results of this screening procedure were unambiguous. Radioactivity determinations were <1,000 cpm for negative wells and >20,000 cpm for positive wells. During the initial screening, random samples that were deemed positive were analyzed by SDS-PAGE to ensure that gp140 was indeed immunoprecipitated. No false positives were detected. A total of 190 hybridomas from nine mice in five fusion experiments were strongly positive in this assay. Of these, 180 were still positive after expansion, and so far 138 have been cloned by limiting dilution. Of the 138 cloned hybridomas, only 15 were derived from mice immunized with monomeric Env. Several factors could account for this relatively low yield. Although approximately the same amount of Env protein was used to immunize each mouse, the

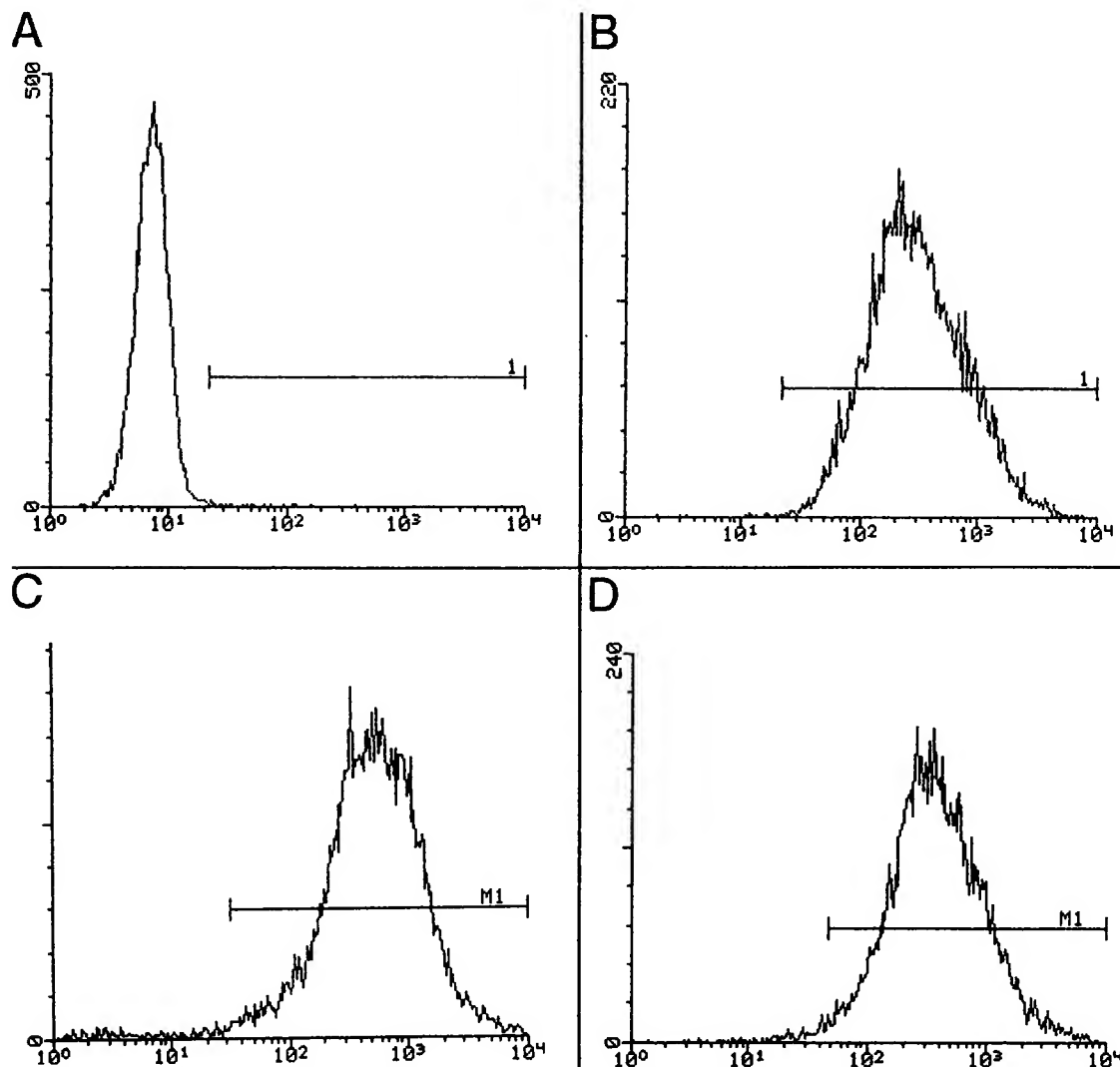


FIG. 2. FACS profiles of MAb. Human T cells chronically infected with HIV-1 IIIB were incubated with hybridoma supernatant followed by goat anti-mouse-fluorescein isothiocyanate and subjected to FACS analysis. (A) Nonspecific mouse IgG; (B) anti-gp120 MAb 110.4; (C) anti-gp120 MAb D34; (D) anti-gp41 MAb D6.

monomer preparation was less pure than the dimer and tetramer preparations, resulting in immunization with some extraneous proteins. A more interesting possibility, however, is that monomeric Env is less immunogenic than oligomeric Env. Studies to address this point directly are under way. Hybridomas, and the MAb that they secrete, were first designated M (monomer), D (dimer), or T (tetramer), depending on the immunogen used, and numbered sequentially after isolation. The IgG subtypes were determined for 128 of the MABs; 62 were IgG1, 52 were IgG2a, 13 were IgG2b, and 1 was IgG3.

Reactivity of MABs to native HIV-1 Env. Because the immunogen used was a noncleavable, truncated form of Env, it was important to ascertain whether the MABs recognized the authentic HIV-1 Env molecule. To do this, approximately 90% of the MABs were screened by FACS analysis for the ability to recognize Env on the surface of cells chronically infected with HIV-1 IIIB. Representative FACS profiles are shown in Fig. 2. A control mouse IgG did not label the cells (Fig. 2A), while a previously described MAB to gp120, 110.4 (Fig. 2B), labeled the cells strongly. The profiles of one new anti-gp120 MAB,

D34, and one new anti-gp41 MAB, D6, are shown in Fig. 2C and D, respectively. At least 80% of the MABs were clearly positive in this assay. Lack of reactivity could be due to a very low titer of antibody or reactivity with an epitope that is unique to the recombinant gp140. However, the fact that the large majority of the MABs tested recognized native HIV-1 Env provides strong evidence that the recombinant, oligomeric gp140 used here faithfully reflects the antigenic structure of the authentic molecule.

Identification of conformation-dependent and -independent MABs. To determine which MABs recognized linear, conformation-independent epitopes, each was screened for the ability to react by Western blot analysis with Env that had been denatured and reduced prior to SDS-PAGE. A representative panel is shown in Fig. 3. We found that 43% of the MABs reacted strongly with Env, 11% reacted very weakly (e.g., D9 and D59), and 46% were completely negative even though they efficiently immunoprecipitated Env. We defined MABs that reacted either very weakly or not at all by Western blotting as conformation dependent. The remaining MABs, which reacted

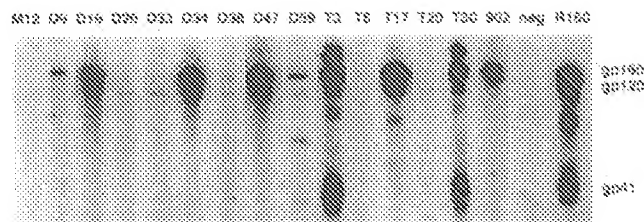


FIG. 3. Western blot analysis of MAb reactivity. A cell lysate of BS-C-1 cells infected with vPE16 was separated by preparative SDS-PAGE (10% gel) and transferred to nitrocellulose. Strips of nitrocellulose membrane were incubated with individual MAbs. All 138 MAbs were tested in this way; a representative set is shown. MAbs were classified as conformation dependent if they showed no reactivity (for example, M12 and D20) or weak reactivity (D9 and D59) and as conformation independent if they were strongly reactive (for example, D19 and D34). The subunit recognized by the conformation-independent MAbs was also determined by this analysis. For example, T3 and T30 recognize gp41, while D19, D34, D47, and T17 recognize gp120.

strongly with denatured Env, were defined as conformation independent. By this criterion, 57% (79 of 138) of the MAbs recognized conformationally sensitive antigenic epitopes, while 43% (59 of 138) recognized linear or conformation-independent epitopes (Table 1). Thus, the immunization and screening approach used here generated a large number of antibodies, more than half of which recognized conformation-dependent epitopes.

Subunit mapping. The epitope recognized by each MAb was mapped initially to either gp120 or the gp41 ectodomain. Mapping of the conformation-independent MAbs was done by

Western blot analysis as shown in Fig. 3. Of the 59 conformation-independent MAbs analyzed, 50 recognized epitopes in gp120 and 9 recognized epitopes in gp41 (Table 1). Subunit mapping of the conformation-dependent MAbs was performed by immunoprecipitation analyses using several different metabolically labeled forms of Env (Fig. 4). These included a cell lysate containing full-length gp160, gp120, and gp41 (Fig. 4A), monomeric gp120 (Fig. 4B), and a gradient-purified gp41 ectodomain fragment derived from vCB-14 (Fig. 4C). The results obtained from a panel of conformation-dependent MAbs is shown in Fig. 4A to C. Of the 79 conformation-dependent MAbs analyzed, 33 mapped to gp120 (Table 1). Many of these anti-gp120 MAbs coprecipitated gp41 in a lysate containing gp160, gp120, and gp41 (for example, D20; Fig. 4), presumably because of noncovalent interactions. However, none of the anti-gp41 MAbs coprecipitated gp120 either because the gp120-gp41 complex was not reactive or because it was dissociated by the MAb.

Of the MAbs that did not immunoprecipitate purified gp120, many immunoprecipitated the purified gp41 ectodomain fragment, indicating that their epitopes reside in the gp41 ectodomain (for example, D12 and D40; Fig. 4C). However, a number of MAbs were unable to immunoprecipitate either purified gp120 or the gp41 ectodomain fragment by itself. These MAbs, such as T6, immunoprecipitated both gp41 and gp160 when they were together in cell lysates (Fig. 4A). As a consequence, it was not possible to determine whether these MAbs recognized epitopes unique to gp160 and coimmunoprecipitated gp41 or whether they recognized oligomer-dependent epitopes present in gp41. To investigate these possibilities further, the MAbs were tested for the ability to immunoprecipitate a chimeric Env protein consisting of HIV-2 gp120 and HIV-1

TABLE 1. Reactivities of 138 MAbs raised against soluble forms of the HIV-1 Env protein^a

MAb	Total	Conformation-independent epitope	Conformation-dependent epitope	V3 loop	CD4 blocking
All					
Total	138	59	79	15/82	19/76
Conformation dependent	79			0/32	19/49
Conformation independent	59			15/50	0/27
gp120					
Total	83	50	33	15/82	19/41
Conformation dependent	33			0/32	19/20
Conformation independent	50			15/50	0/21
gp41					
Total	52	9	43		0/35
Conformation dependent	43				0/29
Conformation independent	9				0/6
Immunogen					
Monomer					
All	15	9	6		3/12
gp120	12	9	3	7/12	3/10
gp41	3	0	3		0/2
Dimer					
All	68	19	49		14/41
gp120	34	14	20	6/33	14/22
gp41	31	5	26		0/19
Tetramer					
All	55	31	24		2/23
gp120	37	27	10	2/37	2/9
gp41	18	4	14		0/14

^a MAbs are classified on the basis of which subunit they recognize (gp120 or gp41), whether they recognize conformation-dependent or -independent epitopes, and by the immunogen used to elicit them. The subunit recognized by three conformation-dependent MAbs made with dimeric gp140 as immunogen has not been determined. Reactivity against the V3 loop and ability to block CD4 binding were determined by peptide ELISA or immunoprecipitation analysis, respectively. Not all MAbs have been tested for reactivity with the V3 loop and CD4 blocking; thus, the total number of positive MAbs over the total number tested is shown.

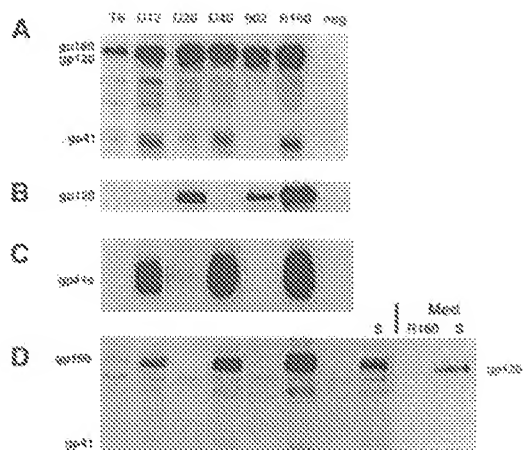


FIG. 4. Subunit mapping of conformation-dependent MABs. The subunit recognized by each conformation-dependent MAb was identified by immunoprecipitation analysis with different forms of Env. For all panels, metabolically labeled Env proteins were produced by infection of BS-C-1 cells with a recombinant vaccinia virus expressing a form of Env. The rabbit polyclonal anti-gp160 antibody R160 (84) was used to show all forms of Env in a given sample. Anti-V3 loop MAb 902 (9) was used as an anti-gp120 positive control, and an irrelevant MAb was used as a negative (neg) control. Each panel shows immunoprecipitation with a different form or preparation of Env as follows: (A) a lysate of cells infected with vSC60 containing wild-type gp160 and the proteolytic cleavage products gp120 and gp41; (B) the medium of cells infected with vPE16 containing monomeric gp120; (C) the ectodomain fragment, gp41s, prepared by sucrose density gradient purification of the medium of cells infected with vCB-14 which expresses cleavable gp140; and (D) a chimera consisting of HIV-2 gp120 and HIV-1 gp41 expressed by vSC64 (7). An extract of cells infected with vSC64 was subjected to immunoprecipitation by the set of MABs as well as by anti-simian immunodeficiency virus monkey serum (S) (donated by V. Hirsch, NIAID). The sharp band migrating slightly below the position of gp120 may be due to β -galactosidase, which is coexpressed by the recombinant vaccinia viruses. The gp160 band appears as a doublet in cells infected with vSC64 (D) as well as in cells infected with vSC50 which expresses HIV-2 Env (not shown). In addition, gp120 (HIV-2) from the medium (Med) of cells infected with vSC64 was immunoprecipitated with R160 and anti-SIV serum (D).

gp41 (vSC64). None of the MABs in the class represented by T6 immunoprecipitated HIV-2 Env (not shown). They did, however, recognize the chimeric Env protein (Fig. 4D), indicating that the epitopes to which they bind are present in the HIV-1 gp41 ectodomain.

Taken together, more than one-third of the MABs derived from immunization with native oligomeric Env protein were directed against epitopes in the gp41 ectodomain. Comparison of the subunit mapping results with the data on conformation dependence revealed that the antigenic structure of gp41 is exquisitely sensitive to conformation. More than 80% (43 of 52) of the MABs to gp41 recognized conformation-dependent epitopes (Table 1). By contrast, the antigenic sites of gp120 appeared to be less sensitive to tertiary structure, since 40% (33 of 83) of the gp120 MABs recognized conformational epitopes (Table 1).

Detailed epitope mapping of MABs. To map the epitopes recognized by the MABs more precisely, a series of C-terminally truncated Env molecules was used. These were expressed either by recombinant vaccinia viruses or by the transient vaccinia virus-T7 system (21). The Env molecules used included full-length gp140 (678 amino acids), two molecules with

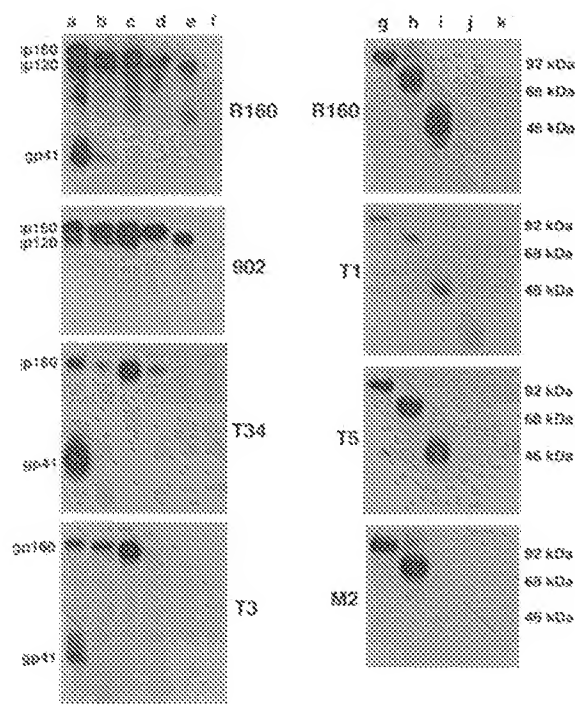


FIG. 5. Epitope mapping of the conformation-independent MABs by Western blot analysis. A set of C-terminally truncated Env molecules expressed by vaccinia viruses was used to determine the region of Env with which each conformation-independent MAB reacted. Extracts of cells expressing the truncated Env proteins were separated by SDS-PAGE (10% gel) and blotted to nitrocellulose. The MABs with reactivity to gp41 were mapped with gp160 (lane a) and four sequential truncations in gp41 containing 747 (lane b), 678 (lane c), 635 (lane d), and 574 (lane e) amino acid residues. The MABs with reactivity to gp120 were mapped with gp120 (lane g) and three sequential truncations in gp120 containing 393 (lane h), 287 (lane i), and 204 (lane j) amino acid residues. The virus vSC8, which does not express Env protein, was used as a negative control (lanes f and k). Antibody names are given between the vertical panels of blots.

sequential truncations in gp41 (635 and 574 amino acids), full-length gp120 (502 amino acids), and three truncated forms of gp120 (393, 287, and 204 amino acids) (17, 19). Mapping of the conformation-independent MABs was performed by Western blotting using extracts of cells expressing the truncated Env molecules. The map location is indicated by the first segment removed that results in loss of reactivity. Thus, T34, T3, T1, T5, and M2 map to locations progressively closer to the N terminus of gp160. Representative examples of Western blots are shown in Fig. 5, and the results for all MABs tested are summarized in Fig. 6. Of the nine anti-gp41 MABs tested, one mapped to amino acids 503 to 574, five mapped to amino acids 575 to 635, and three mapped to amino acids 636 to 678. Of the 50 anti-gp120 MABs to conformation-independent epitopes, 32 mapped to the amino-terminal 204 amino acids, 3 mapped to amino acids 204 to 287, and 15 mapped to amino acids 287 to 393. Somewhat surprisingly, no conformation-independent MABs mapped to the C-terminal region of gp120 (between amino acids 393 and 502) even though antibodies to this region are abundant in human sera (2, 37, 41, 45, 64). This could be due to sequestration of the C terminus of gp120 by interactions with adjoining Env subunits or with gp41. Alternatively, the conformation of the uncleaved gp140 used as immunogen could mask this region.

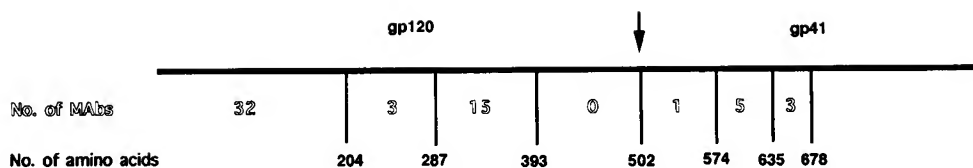


FIG. 6. Compilation of the reactivities of the 59 conformation-independent MABs with truncated Env molecules. Each of the MABs was analyzed for reactivity by Western blot analysis using a set of C-terminally truncated Env molecules as shown in Fig. 5. The number of amino acids in each truncated Env molecule and the number of MABs mapping to each region are given.

Initial mapping of the conformation-dependent MABs was done by immunoprecipitation of metabolically labeled, truncated Env molecules. Of the 39 anti-gp41 MABs tested, 3 efficiently immunoprecipitated the 635-amino-acid Env molecule, indicating that amino acids 635 to 678 are not necessary for antibody recognition. Of the 32 anti-gp120 antibodies tested, only 2 immunoprecipitated the 393-amino-acid gp120 molecule; one of these also immunoprecipitated the 287-amino-acid molecule. However, the inability of a conformation-dependent MAB to immunoprecipitate a truncated form of Env does not necessarily imply that its epitope lies completely or even partially within the truncated area, since the overall structure of the truncated molecules may be significantly different from the native, full-length protein. Other approaches are being used to map the epitopes recognized by our panel of conformation-dependent antibodies.

Reactivity of MABs with the V3 loop peptide. To determine the fraction of MABs directed against the V3 loop generated by immunization with oligomeric Env glycoprotein, a V3 loop peptide ELISA assay was performed with anti-gp120 MABs. We found that 15 of 50 conformation-independent MABs exhibited reactivity with the HIV-1 IIIB V3 loop peptide (Table 1). Of these, two cross-reacted with a V3 loop peptide from the MN strain. Similar cross-reactive antibodies to the V3 loop have been reported previously (25, 27, 60). In addition, an interesting correlation was observed between the oligomeric state of the immunogen used and the frequency with which anti-V3 loop MABs were derived. Of the 15 MABs derived from animals immunized with monomeric gp140, 7 were against the V3 loop. In contrast, only 6 of 68 MABs from animals immunized with dimer and 2 of 55 immunized with tetramer bound to the V3 loop peptide (Table 1). Thus, a far greater proportion of non-V3 loop MABs was obtained when oligomeric Env was used as the immunogen, indicating that the V3 loop may not be a predominant epitope when presented in the context of oligomeric Env. Immunization with oligomeric Env may, as a consequence, generate a greater proportion of antibodies to conserved, conformational epitopes rather than to variable, linear regions of the protein.

CD4 blocking ability of MABs. Unlike antibodies to the V3 loop, neutralizing antibodies which block Env-CD4 binding generally recognize conformationally sensitive epitopes and often recognize the Env from divergent strains (8, 11, 34, 36, 41, 46, 67, 77). We tested a large panel of MABs to both gp120 and gp41 for the ability to block binding of sCD4 to Env. MABs were incubated overnight with gradient-purified, metabolically labeled dimeric gp140 derived from vPE12B. The amount of antibody used was determined to be in excess over the amount of Env. Metabolically labeled sCD4 was then added for 30 min followed by rabbit anti-mouse IgG and protein A-Sepharose beads. Coimmunoprecipitation of sCD4 by the anti-Env MABs was monitored by SDS-PAGE. MAB F105 (67), which blocks CD4 binding, and the anti-V3 loop MAB 902 (10), which does not, were used as controls. A representative set of results is

shown in Fig. 7. MAB D20, for example, efficiently blocked sCD4 binding, while D16 did not. As expected, none of the anti-gp41 MABs blocked binding of sCD4 regardless of their conformation dependence. However, of the 20 conformation-dependent anti-gp120 MABs tested, 19 efficiently blocked sCD4 binding (Table 1). In contrast to conformation-dependent anti-gp120 MABs, none of the 21 conformation-independent anti-gp120 MABs tested blocked CD4 binding. Thus, in this panel of MABs, the ability to block sCD4 binding was restricted to conformation-dependent antibodies to gp120.

DISCUSSION

Characterizing the structural determinants that affect the HIV-1 Env glycoprotein antigenic structure is important for designing Env subunit preparations capable of eliciting broadly cross-reactive, neutralizing antibodies as well as for understanding the humoral response to HIV-1 infection. It is becoming increasingly apparent, for example, that antibodies to conformational epitopes comprise an important component of the immune response to the Env glycoprotein (30, 43, 53, 76). One conformational determinant which may influence Env immunogenicity is its quaternary structure. Like most other viral membrane proteins, Env assembles into oligomers in the endoplasmic reticulum (20), and by analogy with other viral and cellular membrane proteins, assembly is apt to be a prerequisite for further transport (13, 72, 84). To determine the effects of quaternary interactions on Env antigenic structure, we isolated oligomeric Env for immunization and used a screening technique which would allow us to identify antibodies that reacted with nondenatured molecules. To enable purification that would not disrupt the oligomeric structure of the protein, we designed a secreted form of Env which contained all of gp120 and the entire gp41 ectodomain. To prevent dissociation of gp120 from the gp41 ectodomain during isolation, a mutation was introduced to preclude cleavage of the precursor molecule. We and others have shown that cleavage is not essential for conformational maturation and transport (3, 17, 31). The resulting molecule, noncleaved

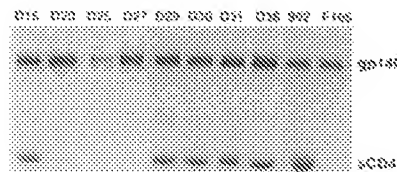


FIG. 7. Analysis of the CD4 blocking ability of MABs. Metabolically labeled, gradient-purified dimeric gp140 was incubated with an excess of MAB overnight. Then trace amounts of metabolically labeled sCD4 were added for 1 h, and immunoprecipitation was performed. MABs F105 and 902 were used to show CD4 blocking and lack of blocking, respectively.

gp140, was secreted primarily in oligomeric form, consistent with reports that have shown the ectodomain of gp41 to be largely responsible for subunit assembly, though interactions between adjoining gp120 subunits may occur (19, 62, 81).

Several lines of evidence suggest that secreted, oligomeric gp140 accurately reflected native Env structure. First, the protein was efficiently secreted. Since misfolded molecules are typically retained in the endoplasmic reticulum and degraded (13, 39, 72), it is unlikely that gp140 was grossly misfolded. Second, gp140 bound sCD4 as well as the conformation-dependent MAb F105 efficiently. Because the CD4 and F105 binding sites in gp120 are discontinuous in nature (44, 61, 67), the gp120 portion of the molecule appeared to be relatively intact. However, the strongest evidence that purified gp140 accurately reflects native Env structure came from analysis of the antibodies raised against it in this study. The large majority of MAbs recognized Env on the surface of chronically infected cells, and 79 of the 138 MAbs recognized conformational epitopes in either gp120 or gp41. These results demonstrate that when oligomeric Env is used for immunization, antibodies to conformation-dependent epitopes are readily generated and suggest that Env tertiary and quaternary structure are important determinants for the humoral response. The large number of MAbs to conformational epitopes reported here lend further support to the idea that oligomeric protein elicits a qualitatively different immune response than monomeric protein (whether native or denatured) and show that it is possible to generate a soluble, oligomeric form of Env which reflects the native structure of the wild-type protein. Several other groups have reported small panels of MAbs produced by immunization with a soluble, secreted form of Env similar to the one that we have used (56, 65). However, relatively few conformation-dependent MAbs were obtained, most likely because of the use of ELISA screening procedures that may preferentially recognize unfolded protein.

Preliminary epitope mapping of the 83 MAbs directed against gp120 revealed several interesting features. We noted that antibodies to the V3 loop comprised a high percentage of MAbs generated from animals immunized and screened with monomeric Env compared to oligomeric Env. Thus, 47% (7 of 15) of the MAbs from animals immunized with monomeric Env bound to epitopes in the V3 loop, compared with 7% (8 of 123) of the MAbs derived from mice immunized with oligomeric protein. This finding suggests that the V3 loop is not as immunogenic in the context of oligomeric protein as it is in monomeric protein. This is consistent with recent studies which have shown that antibodies to the V3 loop are not prevalent in human sera (32, 53). While anti-V3 loop antibodies can be easily generated in the laboratory by immunization with peptides or protein and detected in human sera by using similar material in ELISA assays (25–28, 40, 47, 52, 56, 58–60, 63, 69, 74, 76), the contribution of the V3 loop in the overall immune response to native oligomeric Env may not be great.

The C-terminal 109 residues of gp120 comprised another region to which relatively few antibodies were directed. None of the 50 antibodies to linear gp120 epitopes mapped here, which was surprising since this region contains an immunodominant epitope to which antibodies can be detected in human sera (37, 41, 45, 64). One explanation for our results is that this region may be partially sequestered in the oligomeric molecule through interactions with adjoining gp120 subunits or gp41. In fact, there are reports which suggest that the C-terminal region of gp120 interacts with gp41 (33, 50). Antibodies in human sera to the C-terminal region of gp120 might arise from the presence of shed, monomeric gp120 which was absent from our oligomeric Env preparations. Though present in

human sera, these antibodies may not react or react weakly with oligomeric Env on the surface of virions or infected cells. The fact that these antibodies do not neutralize virus or have weak neutralizing activity (41, 45, 53, 64) is consistent with this model. An alternative explanation for the absence of MAbs to the C terminus of gp120 is that this region is buried in the uncleaved gp140 molecule used for immunization. This possibility cannot be ruled out by the data presented here. Finally, a large number of conformation-dependent anti-gp120 MAbs were found to block sCD4 binding, consistent with reports that indicate gp120 and CD4 interact over a large area representing several discontinuous regions of gp120 (8, 11, 35, 36, 41, 46, 53, 56, 61, 67, 76, 77, 79). By contrast, none of the conformation-independent anti-gp120 MAbs or anti-gp41 MAbs blocked CD4 binding.

Because of the efficiency with which gp140 assembled, it was not possible to obtain large amounts of monomeric gp140. As a consequence, we cannot determine whether the small number of MAbs generated from animals immunized with monomeric gp140 was due to a relatively weak immune response to the monomeric molecule or to differences resulting from less pure protein. While additional studies will be required to determine whether oligomeric Env elicits a stronger humoral response than that seen with monomeric Env, our results do demonstrate several important qualitative differences. A very strong immune response to gp41 was obtained by immunization with oligomeric Env, as judged by the percentage of anti-gp41 MAbs identified. The gp41 ectodomain contained 26% of the total amino acid content of the protein, and 38% (52 of 138) of the MAbs recognized epitopes in this region. Analysis of the anti-gp41 MAbs showed that 82% (43 of 52) recognized conformationally sensitive epitopes, a much higher proportion than that seen with the anti-gp120 MAbs. Furthermore, we have recently found that 60% (21 of 35) of the MAbs which recognize gp41 react more strongly with oligomeric than monomeric Env or, in some instances, are oligomer specific. In addition, some of these MAbs possess neutralizing activity (6). These results suggest that the antigenic structure of gp41 is critically dependent on both Env tertiary and quaternary structure and that the two cannot be dissociated from one another. In addition, we have found that a large number of our MAbs directed against gp120 react more strongly with monomeric than oligomeric protein (6). Thus, immunization with gp120, even if it retains native structure, may lead to the generation of antibodies which react poorly with oligomeric Env on virions and the surface of infected cells.

In summary, we have constructed a soluble, oligomeric form of the HIV-1 Env glycoprotein which reflects native Env structure and elicits a diverse array of antibody reactivities, particularly antibodies to conformational epitopes. The repertoire of antibodies raised against oligomeric Env is qualitatively different than that raised against monomeric Env. It is clear, for example, that Env quaternary structure has significant antigenic implications both in gp41 and gp120. The large number of MAbs that we have generated against gp41, all of which immunoprecipitate native protein, should make it possible to construct a relatively detailed antigenic map of this subunit and to identify regions that are immunogenic and conserved and to which neutralizing antibodies are directed. These findings, coupled with observations that native Env protein elicits neutralizing antibodies more effectively than the denatured molecule (30, 75), strongly argue that taking into account Env quaternary structure will be important in understanding the humoral response to HIV-1 infection and potentially for the design of Env subunit preparations which can effectively elicit broadly cross-reactive, neutralizing antibodies.

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